Alveolar Bone Loss: Mechanisms, Potential Therapeutic Targets, and Interventions

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ABSTRACT

This article reviews recent research into mechanisms underlying bone resorption and highlights avenues of investigation that may generate new therapies to combat alveolar bone loss in periodontitis. Several proteins, signaling pathways, stem cells, and dietary supplements are discussed as they relate to periodontal bone loss and regeneration. RGS12 is a crucial protein that mediates osteoclastogenesis and bone destruction, and a potential therapeutic target. RGS12 likely regulates osteoclast differentiation through regulating calcium influx to control the calcium oscillation-NFATc1 pathway. A working model for RGS10 and RGS12 in the regulation of Ca^{2+} oscillations during osteoclast differentiation is proposed. Initiation of inflammation depends on host cell-microbe interactions, including the p38 mitogen-activated protein kinase (MAPK) signaling pathway. Oral p38 inhibitors reduced lipopolysaccharide (LPS)-induced bone destruction in a rat periodontitis model but showed unsatisfactory safety profiles. The p38 substrate MK2 is a more specific therapeutic target with potentially superior tolerability. Furthermore, MKP-1 shows anti-inflammatory activity, reducing inflammatory cytokine biosynthesis and bone resorption. Multipotent skeletal stem cell (SSC) populations exist within the bone marrow and periosteum of long bones. These bone-marrow-derived SSCs and periosteum-derived SSCs have shown therapeutic potential in several applications, including bone and periodontal regeneration. The existence of craniofacial bone-specific SSCs is suggested based on existing studies. The effects of calcium, vitamin D, and soy isoflavone supplementation on alveolar and skeletal bone loss in post-menopausal women were investigated. Supplementation resulted in stabilization of forearm bone mass density and a reduced rate of alveolar bone loss over 1 yr, compared with placebo. Periodontal attachment levels were also well-maintained and alveolar bone loss suppressed during 24 wk of supplementation.

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INTRODUCTION

This article highlights recent advances in our understanding of mechanisms of pathological bone resorption and their potential clinical implications for treatment of periodontal diseases. Several proteins, signaling pathways, stem cells, and dietary supplements are discussed as they relate to periodontal bone loss and regeneration.

Bone loss occurs in a broad range of human pathologies, including osteoporosis, cancers, and inflammatory diseases, such as periodontitis, and is characterized by disruption of the balance of bone formation and resorption. The mechanisms underlying osteoclast differentiation and activation involve the regulator of G protein signaling (RGS) proteins in osteoclastogenesis. In periodontitis, alveolar bone loss occurs as a consequence of the host immune inflammatory response to oral pathogens, and analysis of recent data, described below, confirms the influence of the p38 mitogen-activated protein kinase (MAPK) signaling pathway in this process.

Key Words

regulator of G protein signaling, p38 MAPK, p38 inhibitors, skeletal stem cells, mesenchymal stem cells, menopause.

Multipotent skeletal stem cells have shown promise for use in bone-regenerative therapies, and research to identify and characterize the stem cell populations present in skeletal tissues is outlined here. Finally, an intervention aimed at reducing post-menopausal skeletal and alveolar bone loss is described. Studies that have investigated the effects of dietary supplementation with calcium, vitamin D, and isoflavone aglycone on alveolar and skeletal bone loss in post-menopausal women have yielded promising results, which are reviewed below.

THE ROLE OF RGS PROTEINS IN THE REGULATION OF BONE RESORPTION

Bone resorption is the unique function of the osteoclast. Osteoclasts are specialized cells that remove mineralized matrix and break up the organic bone components. Calcium is an important signal for osteoclast motility downstream of tyrosine kinase signals (Sanjay *et al.*, 2001). The basolateral membranes of osteoclasts are sensitive to elevated Ca^{2+} and cytosolic Ca^{2+} rises, conditions which result in cell-matrix detachment and cessation of bone resorption (Zaidi *et al.*, 1989). Investigations suggest that regulators of G protein signaling, the RGS proteins (RGS10 and RGS12), play critical roles in regulating Ca^{2+} oscillations and thus osteoclast differentiation (Yang and Li, 2007a, b; Yang *et al.*, 2007, 2013).

How simple Ca^{2+} oscillations regulate cell differentiation is an unsolved question. Some studies have shown that RGS proteins play specific and pivotal roles in controlling Ca^{2+} oscillations and cell differentiation in T-lymphocytes, neurons, and cardiac myocytes (Zeng *et al.*, 1998; Taylor and Thorn, 2001; Ishii and Kurachi, 2003). Currently, there are more than 30 known RGS proteins (Ishii and Kurachi, 2003). Most interestingly, different RGS proteins have different receptor preferences (Xu *et al.*, 1999). Many RGS proteins, including RGS1, RGS2, RGS4, and GAIP, as terminators of the active state of G proteins, can accelerate the GTPase-activating protein (GAP) activity of Gαq (De Vries *et al.*, 2000) to inactivate Ca^{2+} re-uptake and delay Ca^{2+} spikes for several seconds or even minutes. Additionally, the amplitude and frequency of changes in cytoplasmic Ca^{2+} concentrations influence the nature of the cellular response. Rapid oscillations stimulate 3 transcriptional factors [NFAT, Oct/OAP, nuclear factor kappa B (NF-κB)], whereas infrequent oscillations activate only NF-κB. By differentially controlling the activation of distinct sets of transcription factors and the expression of different genes, oscillation frequency may direct cells along specific developmental pathways (Gu and Spitzer, 1995; Fields *et al.*, 1997).

By differential screening, it was shown that both RGS10, the smallest protein in the RGS family, and RGS12, a multi-domain and the largest protein in the RGS family, were prominently expressed in osteoclasts (Yang and Li, 2007b). First, the function of RGS10 was analyzed in osteoclast differentiation and function by the generation and characterization of an RGS10 conventional knockout model. Interestingly, it was found that RGS10 regulates Ca^{2+} oscillations during osteoclast differentiation. RGS10 knockout mice have severe osteopetrosis, impaired Ca^{2+} oscillations, and osteoclast differentiation, which cannot be rescued by

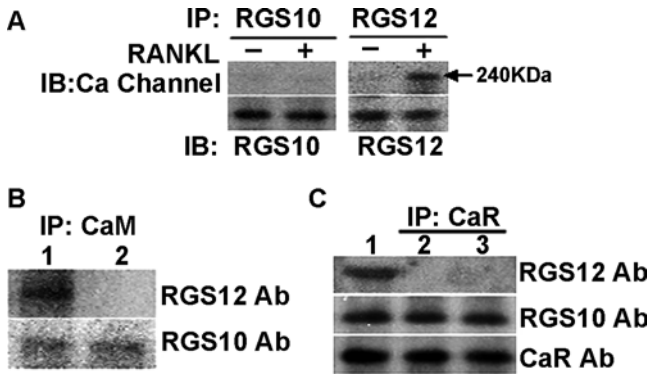

Figure 1. Interaction of RGS10 and RGS12 with the target components (Yang *et al.*, 2007). RAW264.7 cells were induced with RANKL for 96 hr. **(A)** N-type calcium channel binds with RGS12, but not RGS10. **(B)** Lane 1: positive control. RGS10 and RGS12 expressed in RANKL-induced osteoclasts. Lane 2: IP, CaM; IB, RGS12 and RGS10. **(C)** Lane 1: positive controls. RANKL-induced osteoclasts. Lanes 2 and 3: IP, CaR; IB, RGS10, 12, and CaR. RGS, regulator of G protein signaling; RANKL, Receptor activator of nuclear factor kappa-B ligand; CaM, calmodulin; CaR, calcium-sensing receptor.

RGS12 (Yang and Li, 2007a), suggesting that RGS10 and RGS12 likely have different functions in the regulation of Ca^{2+} oscillations during osteoclast differentiation. To further define the function of RGS12 in these processes, a loss-of-function study was performed by a lentivirus-mediated gene silence approach. Knockdown of RGS12 blocked Ca^{2+} oscillations, NFATc1 activation, and osteoclast differentiation (Yang and Li, 2007b). Furthermore, it was found that calcium-sensing receptor (CaR) is expressed in pre-osteoclasts and osteoclasts and that RGS12 interacts with Ca^{2+} channels and CaR in osteoclasts. Other studies showed that CaR plays a pivotal role in controlling the signaling pathway involving CaR activation for osteoclast differentiation (Mentaverri *et al.*, 2006) and that RGS proteins modulate CaR by inhibiting trimetric Gαi protein signaling and phospholipase activities (Handlogten *et al.*, 2001). Additionally, cytosolic Ca^{2+} oscillations were generated mainly by the influx of extracellular Ca^{2+} through multiple channels, which include L- and N-type channels. RGS12 can directly interact with the N-type Ca^{2+} channel through its Phosphotyrosine-binding (PTB) domain and modulates channel activity directly in neuronal cells (Schiff *et al.*, 2000; Richman *et al.*, 2005). To determine if the PTB domain of RGS12 plays an important role in osteoclast differentiation, we constructed overexpression vectors of RGS12 and its PTB domain. After stable transfection of the vectors into RAW264.7 cells and analysis of osteoclastogenesis, we found that ectopic expression of RGS12 or its PTB domain did not fully initiate osteoclast differentiation without the stimulation of receptor activator of NF-κB ligand (RANKL), but it increased the sensitivity of osteoclast precursor cells (RAW264.7 cells) to RANKL (manuscript submitted). These studies demonstrated that the PTB domain of RGS12 plays a very important role in regulating RANKL-induced osteoclast differentiation. To further determine the different functions of RGS10 and RGS12 in osteoclasts, we performed immunoprecipitation assays of RGS10 and RGS12 in RANKL-induced osteoclasts. Interestingly, RGS12

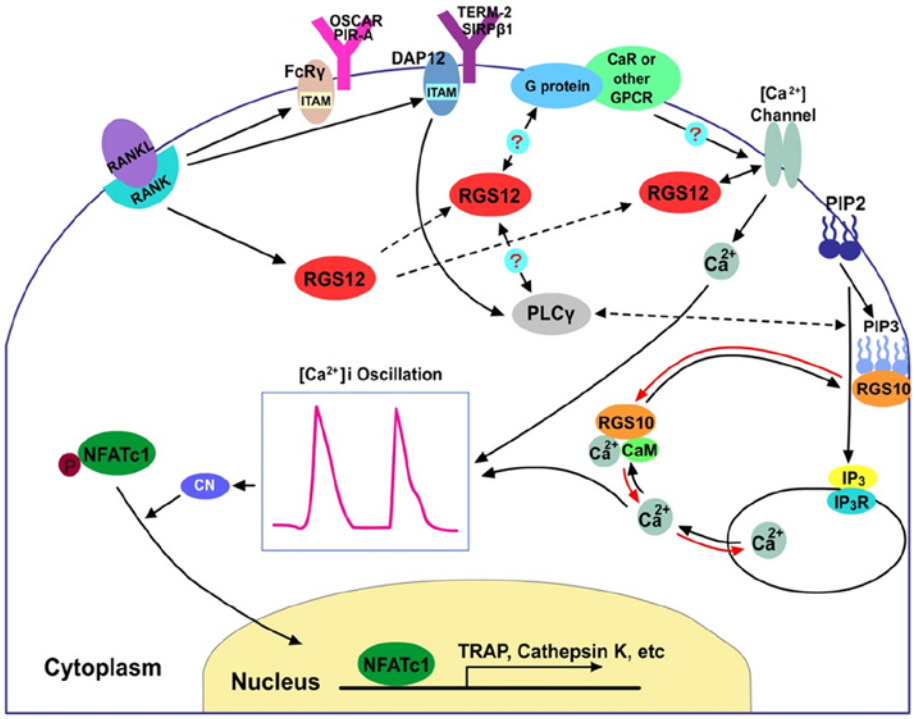

Figure 2. A proposed working model of RGS proteins in the regulation of the Ca^{2+} oscillation-NFATc1 signal pathway for osteoclast (OC) differentiation. We propose that RGS10 and RGS12 play different functions in the regulation of Ca^{2+} oscillations and OC differentiation. RGS10 competitively binds with Ca^{2+}/CaM and phosphatidylinositol (3,4,5)-triphosphate (PIP3) in a Ca^{2+}-dependent manner to internally regulate calcium release from the ER; conversely, RGS12 might interact with Ca^{2+} channels and CaR at the cell membrane to regulate calcium influx during OC differentiation (Yang and Li, 2007a). Thus, RGS10 and RGS12 respectively regulate periodic Ca^{2+} influx and the ER internal release of Ca^{2+} and contribute to the generation and maintenance of Ca^{2+} oscillations and OC differentiation. RGS, regulator of G protein signaling; OC, osteoclast; CaM, calmodulin; PIP3, phosphatidylinositol (3,4,5)-triphosphate; ER, endoplasmic reticulum; CaR, calcium-sensing receptor.

interacted with the α_{1B} subunit of calcium channels, whereas RGS10 did not (Fig. 1A). A previous study showed that RGS10 interacts with calmodulin in a Ca^{2+}-dependent manner (Yang and Li, 2007a). Here, RGS12 did not interact with calmodulin, but RGS10 did (Fig. 1B). Furthermore, it was shown that CaR was expressed in pre-osteoclasts and osteoclasts and that RGS12 bound with CaR, whereas RGS10 did not (Fig. 1C). These results indicated that both RGS10 and RGS12 play roles in regulating Ca^{2+} oscillations; however, they might play different roles in Ca^{2+} oscillations-NFATc1 pathways.

RGS12 function *in vivo* was investigated in a mouse model in which RGS12 was deleted in the osteoclast precursor cell lineage in a CD11b-cre transgenic mouse strain. The RGS12 mutant mice showed increased bone mass and decreased osteoclast number. Deletion of RGS12 impaired osteoclastogenesis and function. Those changes were associated with decreased expression of NFATc1 and the absence of calcium oscillations. Ectopic expression of constitutive active NFATc1 in RGS12-deficient osteoclast precursor cells restored osteoclast differentiation and function, suggesting that RGS12 acts upstream of NFAT2.

Thus, it is clear that RGS12 is a crucial mediator of osteoclastogenesis and bone destruction; RGS12 likely regulates osteoclast differentiation through regulating calcium influx to control the calcium oscillation-NFATc1 pathway. Based on these data, a working model for RGS10 and RGS12 in the regulation of Ca^{2+} oscillations during osteoclast differentiation has been proposed and is presented in Fig. 2. In conclusion, RGS10 and RGS12 may serve as potential and novel drug targets for preventing bone loss in osteolytic bone diseases.

INNATE IMMUNE SIGNALING IN PERIODONTAL INFLAMMATION AND BONE LOSS

Toll-like receptor (TLR) signaling pathways initiate complex signaling pathways following bacterial lipopolysaccharide (LPS) recognition by a macromolecular complex involving CD14, MD2, and TLR4 (reviewed in Li *et al.*, 2014). In response to LPS, a complex formation triggers MyD88 and, in turn, recruits IRAK and TRAF6. The phosphorylated IRAK/TRAF6 complex then dissociates from the receptor complex to a new complex with transforming growth factor β-activated kinase (TAK1) along with TAK-1 and -2 binding proteins (TAB-1 and -2), which phosphorylate TAK1. TAK1, in turn, phosphorylates both the inhibitor of NF-κB (IκB)-kinase complex (IKK complex) and mitogen-activated protein kinase (MAPK) kinases-3 and -6 (MKK3, MKK6). The IKK complex then phosphorylates IκB, which allows NF-κB transcription factors (p50/p65) to translocate to the nucleus and bind to the promoter regions of many pro-inflammatory cytokine and chemokine genes and activate their transcription. Similarly, MKK3/6 can phosphorylate p38 and c-jun N-terminal kinase (JNK) MAPK to activate activator protein-1 (AP-1) transcription factors (TFs) and initiate gene expression. In addition, p38 can phosphorylate RNA-binding proteins, which can stabilize cytokine mRNA and thus amplify cytokine production.

There is abundant evidence indicating that the adaptive immune responses, including humoral and cellular aspects, are fundamentally important in mediating the host responses to micro-organisms of the oral biofilm and also in tissue destruction associated with periodontal diseases (Baker *et al.*, 2002; Kawai *et al.*, 2007). Even though cells participating in the adaptive immune response are considered to be a primary source of cytokines leading to bone resorption (Kawai *et al.*, 2006), evidence demonstrating that bone resorption may occur in the absence of B and T cells has been established (Baker *et al.*, 2002). To understand how inflammation is initiated in response to micro-organisms, it is necessary to focus on the primary interactions with host cells responsible for innate immunity. In this

sense, TLR signaling is considered the most important interface between the host and the microbes.

Adjuvant therapies for periodontitis that modulate the immune response have been used for decades. Approaches to block the progression of inflammatory bone loss observed in periodontitis include host modulation of matrix metalloproteinases (MMPs), cyclooxygenase-2 (COX-2), and arachidonic acid metabolites. However, these therapies target singular mechanisms of alveolar bone destruction. Cytokines are well-known to compensate for one another, thereby limiting the effects of cytokine-specific inhibitors. Alternatively, targeting a common regulatory mechanism for multiple cytokines may repress periodontal disease progression and improve treatment response. Therapeutic modulation of signaling pathways can affect various genes, depending not only on the pathway but also on the relative position targeted for inhibition in the signaling cascade.

Activation of p38 MAPK signaling mediates inflammatory cytokine expression such as IL-1β, IL-6, and TNF-α either directly or indirectly. In addition, the role of MAPK signaling is critical to link the innate and adaptive immune responses (reviewed in Huang *et al.*, 2009). The significance of p38 MAPK signaling during innate immune responses in periodontal disease progression has been shown *in vivo* in a rat model, in which orally active p38 inhibitors reduced periopathogenic LPS-induced bone destruction (Kirkwood *et al.*, 2007). Bone area and volumetric analysis by micro–computed tomography (μCT) indicated significant bone volume loss with LPS treatment, but this was blocked with both doses of p38 inhibitor (Fig. 3). Histological examination indicated significantly fewer osteoclasts adjacent to the areas of active bone resorption, including the periodontal ligament area, and a significant decrease in IL-6, IL-1β, and TNF-α expression in p38 inhibitor-treated groups compared with LPS-treated rats. This proof-of-principle study supports the role of p38α MAPK inhibitors to potentially ameliorate LPS-induced alveolar bone loss.

However, p38 inhibitors have failed as therapeutics in clinical settings because of unacceptable safety profiles, including toxicity, significant off-target effects, and lack of oral bioavailability (Dominguez *et al.*, 2005). To date, no p38 inhibitor has been approved for clinical usage. However, the targeting of downstream substrates of p38 MAPK and factors that regulate transcription, nuclear export, mRNA stability, and translation could be a promising therapeutic alternative for inhibiting inflammatory gene expression to treat various inflammatory diseases. As a direct substrate of the stress-activated MAPK p38α and β, MAPK-activated protein kinase 2 (MAPKAPK-2, MK2) is regulated exclusively by p38α/β. MK2 should constitute a more specific target than p38, with potentially fewer side effects, because MK2 acts on a more limited downstream substrate repertoire compared with p38. However, targeting MK2 with small molecular inhibitors has proved to be difficult because of the relatively planar ATP-binding site critical for MK2 activation/inhibition.

Proof-of-concept studies were performed with an RNA interface strategy to silence MK2 for control of periodontitis progression. *In vivo* studies used the rat LPS-induced experimental periodontitis model to elucidate further the role of MK2 in the pathogenesis of periodontitis and to evaluate the therapeutic potential of targeting MK2 in periodontal disease. The protection of MK2 small interfering RNA from alveolar bone loss in an LPS-induced periodontitis model was also verified by μCT analysis (Fig. 2; Li *et al.*, 2011). This work confirmed the role played by MK2 in a preventive model of experimental periodontitis, suggesting a novel target for controlling periodontal inflammation.

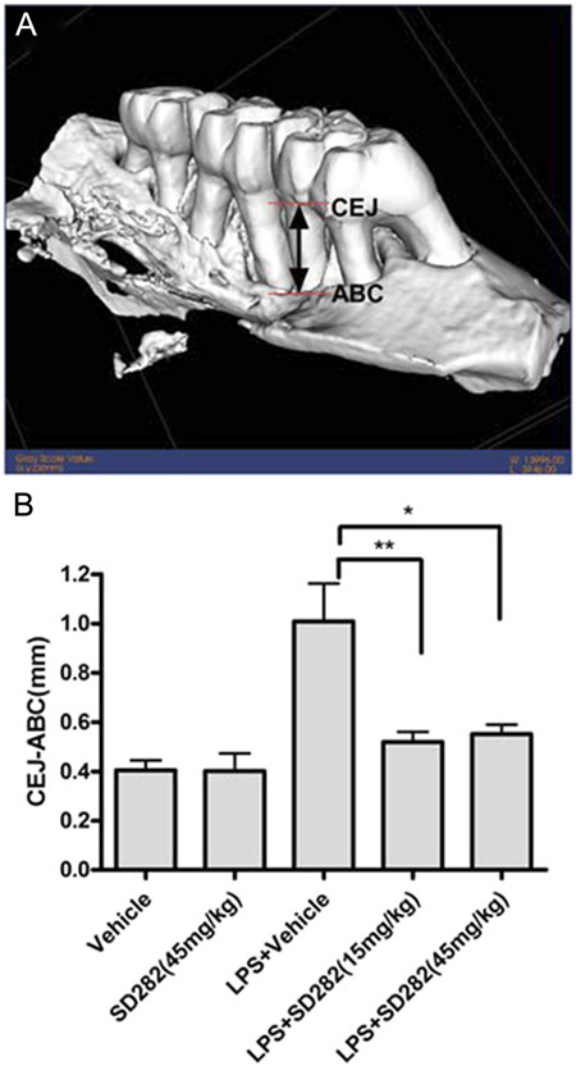

Figure 3. *A. actinomycetemcomitans* LPS induces significant linear bone loss, which is blocked by p38 inhibitor, SD282. **(A)** Reformatted μCT isoform display from eight-week *A. actinomycetemcomitans* LPS-injected rat maxillae exhibits dramatic palatal and interproximal bone loss. Landmarks used for linear measurements were the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC). Differences between these anatomic locations in defined locations of 2-D displays determined alveolar bone loss. **(B)** Linear bone loss as measured from the CEJ to ABC (mean ± SEM). Significant bone loss ($p < .01$) was observed between control (n = 6) and *A. actinomycetemcomitans* LPS-injected rats (n = 12). Significant reduction of LPS-induced periodontal bone loss (**$p < .01$ for SD-282 [15 mg/kg; n = 8] and *$p < .05$ for SD-282 [45 mg/kg; n = 8]) (Kirkwood *et al.*, 2007). Reproduced with permission.

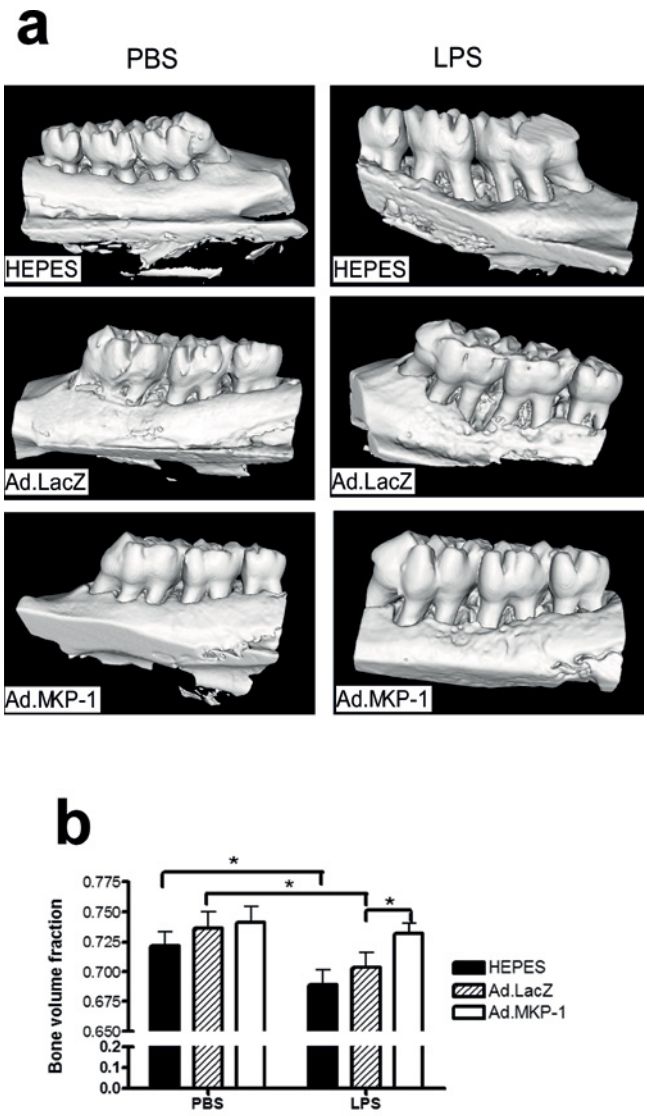

Figure 4. MKP-1 gene transfer alleviated bone resorption in rats after LPS challenge. Eight-week-old male Sprague-Dawley rats (17 rats/group) were injected with either Ad.MKP-1, or Ad.LacZ control (1 x 10^9 pfu in 4 μL), or with HEPES-buffered saline (4 μL). Forty-eight hr after the adenovirus injection, the rats were injected with 2 μL of either 20 μg of LPS (from *A. actinomycetemcomitans*) or phosphate buffered saline (PBS) 3 times a wk for 4 wk. **(a)** Representative microcomputed tomography images of rat maxillae from indicated treatment groups. **(b)** Volumetric analysis of bone loss levels (n = 7 for PBS groups, n = 10 for LPS groups, $*p < .05$) (Yu *et al.*, 2011). Ad, recombinant adenovirus. Reproduced with permission.

Since MKP-1 plays a crucial role in decreasing inflammatory cytokine biosynthesis, we explored the role of MKP-1 in an experimental periodontitis model, where wild-type and *Mkp-1* null mice received *Aggregatibacter actinomycetemcomitans* LPS. Results indicated that, in LPS-injected *Mkp*-$1^{-/-}$ mice, significantly greater bone loss occurred with more inflammatory infiltrate in the periodontal areas injected with LPS and a significant increase in osteoclastogenesis compared with that at *Mkp*-$1^{-/-}$ control sites or in wild-type littermates. MKP-1 displayed a protective response in this more chronic model of inflammation and bone loss (Sartori *et al.*, 2009).

In gain-of-function experiments, MKP-1 was able to dephosphorylate all 3 MAPKs *via* MKP-1 gene transfer with recombinant adenovirus MKP-1 in rat macrophages (Yu *et al.*, 2011). Furthermore, *in vivo* MKP-1 gene transfer in an experimental periodontal disease model attenuated bone resorption induced by LPS (Fig. 4; Yu *et al.*, 2011). Histological analysis confirmed that periodontal tissues transduced with Ad.MKP-1 exhibited fewer infiltrated inflammatory cells, fewer osteoclasts, and reduced cytokines compared with controls. Currently, studies have been initiated to determine if MKP-1 small-molecule agonists have utility in the management of periodontal disease progression.

Taken together, these studies indicate the importance of the p38 MAPK/MKP-1 axis in the development of immune responses that contribute to LPS-induced alveolar bone loss. These signaling intermediates may be key therapeutic targets to control alveolar bone loss associated with chronic periodontitis.

EXPLORING THE LOCATION AND THE MOLECULAR REGULATION OF THE INTRAMEMBRANOUS BONE STEM CELL NICHE

Skeletal Stem Cells of the Bone Marrow

Adult stem cells are defined as multipotent if they are able to generate various types of specialized tissues and if they show transgermal plasticity (Bianco *et al.*, 2001). The most stringent definition for multipotency specifies the ability of a single cell to give rise to a progeny of cells that make a specific tissue or part of it (Bianco *et al.*, 2008). Multipotent stem cells that originate from the mesenchyme are defined as mesenchymal stem cells (MSCs). The existence of multipotent MSCs within the bone marrow has been hypothesized for many years. However, since cells isolated from bone marrow have only been proven to give rise to cell progenies found within the surrounding anatomic sites, such as osteoblasts for bone, chondrocytes for cartilage, and adipocyte or adventitial reticular cells for bone marrow stroma (Bianco *et al.*, 2008), they are more properly categorized as skeletal stem cells (SSCs).

Traditionally, bone-marrow-derived SSCs are defined by their *in vitro* adhesive characteristics, ease of culture expansion, and ability to differentiate into bone, cartilage, and fat (Y Jiang *et al.*, 2002; Caplan, 2005; Bianco *et al.*, 2008). They also express markers such as MCAM, STRO-1, or Mx-1 (Bianco *et al.*, 2008; Park *et al.*, 2012) and have been shown to have therapeutic potential after culture expansion for numerous applications, including bone and periodontal regeneration (Yang *et al.*, 2010), inflammatory conditions (Bouffi *et al.*, 2010; Perez-Simon *et al.*, 2011), and cardiovascular diseases (Garbern and Lee, 2013). Bone-marrow-derived SSCs may not be the only SSC population present in the skeleton. It is possible that SSCs or MSCs reside in other skeletal tissues, such as cortical bone, trabecular bone, cartilage, and the periosteum. Among these tissues, the periosteum has received most attention as a potential source of stem cells.

Skeletal Stem Cells of the Periosteum

Several studies have shown that the periosteum has osteogenic cells within its cambium layer (Lin *et al.*, 2014). Bone-grafting studies demonstrated that the expansion of a progenitor cell population from the periosteum largely accounts for the formation of cartilage and bone within the callus (Lin *et al.*, 2014). Lineage-tracing analysis further showed that cells expressing the paired-related homeobox transcription factor Prx1 (also known as Prrx1) during embryogenesis give rise to progenies resident within the periosteum and the growth plate (Almeida *et al.*, 2013). Prx1 is a transcription factor required for proper skeletal development (Martin *et al.*, 1995) and is expressed in high quantities during limb bud formation (Logan *et al.*, 2002) and in the periosteum of endochondral bones of adult mice (Kawanami *et al.*, 2009). Cells derived from the primordial Prx1+ cells are essential for periosteal-mediated fracture repair in adult long bones (Tsuji *et al.*, 2006), and cells expressing Prx1 isolated from the post-natal periosteum have features characteristic of osteogenic progenitor cells (Kawanami *et al.*, 2009). Analysis of these data suggests that Prx1 may represent a useful marker for periosteal stem cells. Although no further studies have been performed in support of the stem qualities of periosteal-derived Prx1+ cells, stem-like cells derived from the periosteum have been shown to be clonogenic; to differentiate in osteoblasts, chondrocytes, and adipocytes (Ferretti *et al.*, 2012); and to be able to generate skeletal muscle cells when single-cell-derived clonal cell populations are transplanted *in vivo* (De Bari *et al.*, 2006). Thus, SSCs exist within the periosteum, and they may have characteristics observed in MSCs.

It may be concluded that at least 2 different SSC populations exist within the appendicular skeleton. Therefore, the existence of 2 different stem cell niches, 1 intra-marrow and 1 periosteal, can be hypothesized. Further studies are needed to analyze the interplay that takes place between the 2 SSC populations and their respective niches.

Although studies aimed at characterizing SSCs or MSCs have traditionally been performed on tissues obtained from long bones, recent years have seen intensive research into SSCs/MSCs and their niches within craniofacial bones.

In Search of the Intramembranous Bone Stem Cell and Stem Cell Niche

In a study designed to identify the cell lineage that contributes to the regeneration of the injured adult bone, Leucht *et al.* (2008) found that bone defects created in the neural-crest-derived mandible heal with neural-crest-derived cells, whereas bone defects created in the mesoderm-derived tibia heal with mesoderm-derived cells. They further showed that these 2 populations of cells have different regenerative potentials. Thus, since craniofacial bones are derived from both neural crest and mesoderm (X Jiang *et al.*, 2002), it is possible that 2 populations of SSCs, 1 neural-crest-derived and 1 mesoderm-derived, exist in craniofacial bones. In support of this hypothesis is the study by Bhattacherjee *et al.* (2007), who proved that neural-crest-derived and mesoderm-derived cells isolated from the first brachial arch of an embryo have different molecular fingerprints. However, whether these 2 different populations of stem cells really exist in the adult craniofacial bones, and whether they can interact or need to maintain their specific and separate niches and regenerative domains, has yet to be proven. Further studies are needed to identify and characterize the features of the intramembranous bone stem cells and their niches, with the ultimate goal of designing more effective strategies for the regeneration of the craniofacial bones.

EFFECTS OF A CALCIUM, VITAMIN D, AND SOY ISOFLAVONE DIETARY SUPPLEMENT ON ALVEOLAR AND SKELETAL BONE LOSS IN POST-MENOPAUSAL WOMEN

Post-menopausal loss of bone mass and low dietary calcium are risk factors for periodontal disease (Kribbs *et al.*, 1990; von Wowern *et al.*, 1994; Wactawski-Wende *et al.*, 1996; Nishida *et al.*, 2000). There is a need for intervention that will reduce the rate of both skeletal and alveolar bone loss in post-menopausal women. Calcium, vitamin D, and soy isoflavones have been suggested as treatments to reduce skeletal bone loss, while calcium and vitamin D supplementation have been shown to reduce tooth loss in post-menopausal women (Krall *et al.*, 1994; Wactawski-Wende *et al.*, 1996; Nishida *et al.*, 2000; Wactawski-Wende, 2001; Boonen *et al.*, 2006). Several studies have examined the effects of phytoestrogen supplements on bone mineral density (BMD) and other measures of bone health (Chen *et al.*, 2004; Gallagher *et al.*, 2004; Lydeking-Olsen *et al.*, 2004). Two clinical investigations were designed to determine whether dietary supplementation with a calcium, vitamin D, and soy isoflavone preparation was safe and effective in reducing skeletal and alveolar bone loss at periodontal sites and in maintaining periodontal attachment level in post-menopausal women (Inagaki *et al.*, 2003; Grossi *et al.*, 2004). These 2 studies were also anticipated to help determine the optimal calcium, vitamin D, and soy isoflavone preparation for post-menopausal women.

The first study was designed as a randomized, placebo-controlled, double-blind trial with 3 groups: a placebo group; a single-dose group receiving a single daily dose consisting of 500 mg of calcium and 10 mg of soy isoflavone aglycone; and a double-dose group receiving 1,000 mg of calcium and 20 mg of soy isoflavone aglycone (Grossi *et al.*, 2004). Both supplementation groups also received 110 international units (IU) of vitamin D. In total, 75 naturally post-menopausal women, not using hormone replacement therapy or bone-sparing agents and consuming less than 1,000 mg of calcium intake daily, were eligible for the study.

Skeletal BMD assessed by dual-energy x-ray absorptiometry (DEXA) and change in forearm BMD over 1 yr is shown in Fig. 5. Total forearm BMD was statistically significantly reduced in the placebo group by 1.34% ($p = .0008$). However, the change in BMD did not reach statistical significance in the single-dose group, which showed a reduction of 0.68% ($p = .13$). In the double-dose group, BMD was reduced by 0.86% ($p = .58$). The results show that forearm BMD is stabilized by supplementation over a one-year period. There were no changes in the rate of loss of BMD in other skeletal areas. Alveolar crestal height (ACH) changes are shown in the Table, and it can be seen that the mean

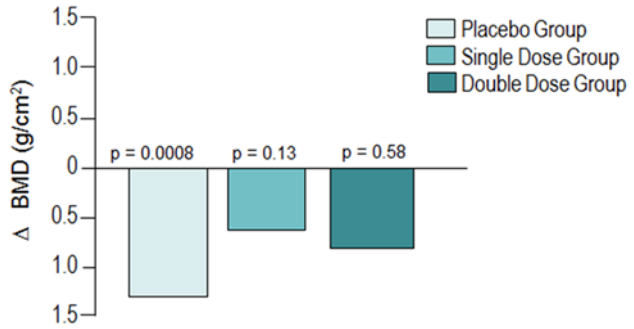

Figure 5. Percentage change in forearm bone mineral density (BMD) over 1 yr in post-menopausal women receiving daily supplementation with vitamin D, calcium, and isoflavone aglycone or placebo. Single dose = 500 mg calcium, 10 mg soy isoflavone; double dose = 1,000 mg calcium, 20 mg isoflavone. Both supplementation groups also received 110 international units (IU) of vitamin D (Grossi *et al.*, 2004).

ACH changes at 1 yr were not significant for any of the groups. However, when an analysis was made of sites exceeding the threshold of ACH change (> 0.522 mm, based on the individual's standard deviation of repeated measurements), the placebo group was found to show at least threshold-level bone loss at 6.59 ± 5.24% of sites. In contrast, the single-dose group experienced threshold or greater ACH change at 3.65 ± 3.57% of sites, and the double-dose group at 4.27 ± 4.88% of sites. The single- and double-dose groups, taken individually, did not show any statistically significant differences relative to either the placebo group or each other. However, when the results of the supplementation groups were pooled for evaluation of the total impact of supplementation *vs*. no supplementation on ACH change, the percentage of sites with threshold-level loss was 3.97 ± 4.25%, which was significantly lower than that in the placebo group ($p = .027$).

The second study was conducted in Aichi Gakuin University Dental Hospital (Inagaki *et al.*, 2003). Eighty-five post-menopausal women receiving periodontal maintenance were randomly assigned to one of the following treatment groups: placebo, calcium (500 mg/day), soy isoflavone aglycone (10 mg/day), and combination treatment with calcium (500 mg/day) plus soy isoflavone aglycone (10 mg/day). Probing pocket depth (PPD), clinical attachment level (CAL), plaque index (PI), and matrix metalloproteinase-8 (MMP-8) in gingival crevicular fluid (GCF) were measured at baseline, 12, and 24 wk following commencement of dietary supplementation.

In all 4 groups, sites with 3 mm or more CAL at baseline showed a significant improvement ($p < .01$). The mean CAL gains from baseline to 24 wk in the calcium, soy-isoflavone, and combination groups were 0.55 mm, 0.61 mm, and 0.48 mm, respectively, and were significantly greater than the mean CAL gain of 0.30 mm in the placebo group. The rate of change was significantly lower in the combination group (0.3 mm) compared with that in the placebo group (2.7 mm). Furthermore, MMP-8 levels of soy isoflavone and combination groups were significantly reduced from baseline to 12 and/or 24 wk ($p < .05$ and $p < .05$, respectively).

In conclusion, both studies show that calcium, vitamin D, and soy isoflavone supplementation was well-tolerated, and the individuals complied in taking it during the test periods. There were few adverse events noted and none that could be related to the supplement. The first study reveals that BMD loss at the forearm was reduced significantly and ACH loss was also reduced significantly in the supplementation groups. The second study shows that alveolar bone loss was suppressed and periodontal attachment level was well-maintained in the supplementation group in post-menopausal women.

CONCLUSION

Analysis of the data presented in this paper sheds new light on the complex processes that culminate in bone destruction and suggest novel possibilities for therapeutic intervention. The role of RGS12 in mediating osteoclast differentiation and activation has been established. Further research is being performed to identify the function and mechanism of RGS12 in osteoblast differentiation and function, and the coupling between osteoblasts and OCs. Similarly, the proven involvement of the p38

Table 1. Interproximal Alveolar Crestal Height (ACH) Changes in Post-menopausal Women Receiving Daily Supplementation* with Vitamin D, Calcium, and Isoflavone Aglycone or Placebo

Variables	Placebo (n = 25)	Single Dose (n = 22)	Double Dose (n = 25)	*p* value
ACH change over 1 mm (mean ± SD)	–0.020 ± 0.110	0.025 ± 0.078	–0.009 ± 0.111	NS
Sites exceeding threshold (ACH ≥ 0.522 mm) % (mean ± SD)	6.59 ± 5.24	3.65 ± 3.57	4.27 ± 4.88	.078
Sites exceeding threshold (ACH ≥ 0.522 mm) % (mean ± SD)	6.59 ± 5.24	3.97 ± 4.25		.027

*Single dose = 500 mg calcium, 10 mg soy isoflavone; double dose = 1000 mg calcium, 20 mg isoflavone. Both supplementation groups also received 110 international units (IU) of vitamin D (Grossi *et al.*, 2004).

MAPK signaling pathway in LPS-induced alveolar bone loss means that several signaling intermediates may be candidate therapeutic targets.

Tissue regeneration of bony defects by the use of host multipotent stem cells has long been a clinical goal; however, much is still to be learned about skeletal stem cell populations, their niches and characteristics. Further research will uncover more about the regenerative potential and possible clinical utility of stem cells in periodontal disease.

The results from studies of calcium, vitamin D, and isoflavone supplementation strongly suggest that this nutritional intervention may be of value in attenuating alveolar bone loss and in maintaining periodontal attachment levels in postmenopausal women.

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